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13. ABSTRACT (Maximum 200) The role of the ErbB3 protein in breast cancer cell proliferation was examined. In previous studies, we investigated the signal transduction mechanism of the ErbB3 protein, which cooperates with ErbB2 in the formation of a coreceptor for the growth factor heregulin. We demonstrated that the ErbB3 protein, although possessing a protein tyrosine kinase (PTK) homology domain, is in fact devoid of intrinsic PTK activity. In the second year of funding, we have demonstrated the critical dependence of heregulin signalling upon the PTK activity of ErbB2. To this end, the PTK domain of ErbB2 was mutated, such that its intrinsic kinase activity was abolished, and this mutant ErbB2 protein was expressed along with wild-type ErbB3 in cultured cells. Our results indicated that the ErbB2 mutation abolished heregulin signal transduction. We also characterized the interaction of ErbB3 with the p85 subunit of phosphatidylinositol 3-kinase, an enzyme known to be involved in heregulin signalling. By use of the yeast two-hybrid system, we mapped the sites of interaction between ErbB3 and p85. Finally, we have pursued the cloning of the heregulin gene promoter, in effort to understand the regulation of heregulin expression in breast cancer cells.				
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FOREWORD

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John J. Holland 9/28/96
PI - Signature Date

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INTRODUCTION

Breast cancer cells have been observed to express abnormally high levels of receptor proteins in the ErbB family, which includes the EGF receptor, ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (1-5). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (6). Given that these receptors activate mitogenic signalling pathways, it is possible that they play a role in the abnormal proliferation of breast cancer cells. The polypeptide heregulin (or Neu differentiation factor) (7) is secreted from breast cancer cells (8), and has been shown to activate ErbB2, ErbB3 and ErbB4 receptor proteins (9-12). Indeed, recent work has shown that ErbB2 and ErbB3 function together as a coreceptor for heregulin (13). Studies completed in the first year of funding had demonstrated that the ErbB3 protein, unlike other ErbB family members, is actually devoid of intrinsic protein tyrosine kinase (PTK) activity. Apparently, in the ErbB2/ErbB3 coreceptor, the ErbB3 protein binds the heregulin peptide, whereas the ErbB2 protein provides the PTK activity necessary for signal transduction.

The purpose of the proposed research is to examine the mechanisms by which the ErbB3 protein is activated in breast cancer cells, and to determine if the ErbB3 protein influences specific mitogenic signal transduction pathways involved in abnormal cellular proliferation. Work performed in the second year of funding addressed fundamental questions about ErbB3 receptor protein function. (1) By what mechanism is PTK activity activated in the ErbB2/ErbB3 heregulin coreceptor complex? (2) What are the cellular targets for the activated ErbB3 protein? (3) What is the mechanism of interaction between the ErbB3 protein and the known signal transduction molecule phosphatidylinositol (PI) 3-kinase? A final question concerned the polypeptide heregulin, which is secreted by breast cancer cells and is possibly involved in their aberrant proliferation. (4) How does the heregulin gene promoter function to regulate heregulin expression in various cell types?

BODY

I. Molecular Cloning and Analysis of the Heregulin Gene Promoter

As detailed in the previous Progress Report, the experiments of the original Specific Aim 1 were to be superseded by a molecular biological analysis of the heregulin gene promoter. The heregulins constitute a large family of polypeptides apparently encoded by a single gene and generated by alternative mRNA splicing (7,8). Expressed conspicuously in the peripheral nervous system (14), the heregulin gene has critical functions in development (15) and is also secreted by breast cancer cells and Ras-transformed fibroblasts (7,8). Able to stimulate the activation of mitogenic signalling by ErbB family receptor proteins, the heregulins could conceivably function as transforming growth factors and hence be involved in the abnormal proliferation of breast cancer cells. One goal of the previous year of research was to clone and characterize the promoter of the heregulin gene. Such analysis of the heregulin gene promoter would give us a better understanding of the regulation of heregulin gene expression in the context of both normal and transformed cells.

Two general approaches were used in efforts to isolate the heregulin promoter: polymerase chain reaction (PCR) amplification, and classical library screening. In the PCR approach, primers complementary to the known 5'-sequence of the heregulin cDNA were used to amplify human genomic DNA in five different PCR-ready libraries provided in a Promoter Finder kit (Clontech). These libraries were restriction-digested genomic DNA with ligated 5'-prime adapters that served as anchors for the provided anchor-specific primers. In theory, PCR amplification of the library DNAs with heregulin-specific reverse and anchor-specific forward primers would yield products corresponding to the upstream sequences of the heregulin gene, and the sensitivity and specificity of the amplification would be enhanced by the use of nested pairs of gene-specific and anchor-specific primers. In the course of these efforts, a variety of nested heregulin-specific primers, 6 different primer pairs in total, and a variety of PCR amplification conditions were tested. Although a large number of PCR products were ultimately generated and subject to partial DNA sequence analysis, none contained any recognizable element of the 5'-heregulin cDNA, as would be expected in an authentic promoter fragment. Hence, this strategy failed to yield the upstream heregulin gene sequences. While the reasons for the failure of the PCR strategy are unclear at this time, two possibilities have been considered.

First it was noted that the 5'-end of heregulin exon 1 is extremely GC rich, which could explain our failure to PCR-amplify genomic DNA proximal to this exon. Second, it is possible that the reported sequence of heregulin exon 1 (16) is in fact not that of heregulin exon 1, but that of a second exon downstream of an as yet unidentified exon 1. In support of this latter hypothesis is the fact that the splicing of heregulin cDNAs is known to be highly complex, and that only relatively recently were splice-variants containing the presumed exon 1 identified (16).

A second approach to the isolation of heregulin upstream regulatory DNA sequences involved classical library screening by plaque hybridization. Here we used a previously isolated heregulin cDNA probe generated by reverse transcriptase-PCR amplification of exons 7, 8 and 9 with heregulin-specific primers to screen an SK-BR3 human breast cancer cell cDNA library and clone the most 5'-prime elements of heregulin cDNA (exon 1). Knowledge of the true nucleotide sequence of the most 5'-end of heregulin cDNA would be subsequently used to design new primers for the PCR amplification of the heregulin promoter. Alternatively, isolated heregulin cDNAs containing exon 1 could be used in the screening of genomic DNA libraries to allow the isolation of upstream promoter elements. cDNA library screening with the heregulin probe is still in progress, and has not yet yielded clones containing upstream exons of heregulin cDNA. However, as the heregulin cDNA is known to be expressed in SK-BR3 breast cancer cells, we anticipate that we can soon successfully isolate the true heregulin exon 1.

II. Protein Tyrosine Kinase Activation in the ErbB2/ErbB3 Coreceptor

In the previous year of funding and in pursuit of Specific Aim 3, we obtained evidence that the ErbB3 protein, while possessing a protein tyrosine kinase (PTK) homology domain in its predicted cytoplasmic domain, is actually devoid of intrinsic PTK activity. Hence the ErbB3 protein is functionally distinct from other ErbB family members, which each possess intrinsic PTK activity. The results of these studies have led to the submission of a manuscript that has recently been accepted pending revision [(17), see Appendix]. Although we have failed to detect PTK activity in the ErbB3 protein, it might be argued that this activity is only expressed in the context of a heterodimeric receptor complex, such as the ErbB2/ErbB3 heregulin coreceptor. To this end we have in the past year generated, by site-directed mutagenesis, mutant ErbB2 and ErbB3 receptor

proteins, each incorporating a single Lys→Met substitution in the predicted PTK domain active site. This mutation is known to abolish the catalytic activities of a variety of PTK enzymes, and would be expected to abolish that of the ErbB2 protein and any PTK activity expressed by ErbB3. The ErbB2 and ErbB3 proteins, both wild-type and kinase-deficient mutants, were expressed in various combinations in COS7 cells via the transfection of their cDNAs in a cytomegalovirus promoter-driven vector. The following observations were made. First, wild-type ErbB2 and ErbB3 together constituted a functional heregulin receptor protein in which the ErbB3 protein, in response to heregulin, became phosphorylated on tyrosine residues and bound to phosphatidylinositol (PI) 3-kinase. Whereas the kinase inactivating Lys→Met mutation in ErbB3 did not abolish heregulin-dependent phosphorylation and PI 3-kinase association, this mutation in ErbB2 did abolish receptor phosphorylation and signal transduction. We concluded that, in the context of the ErbB2/ErbB3 receptor heterodimer, PTK activity is provided by the ErbB2 protein, consistent with our previous finding that the ErbB3 protein has no intrinsic PTK activity. The function of the ErbB3 protein in the coreceptor is apparently in the presentation of a high-affinity heregulin binding site.

The results of these studies shed significant light upon the mechanism of activation of the ErbB2/ErbB3 heregulin coreceptor, by conclusively demonstrating ErbB2/ErbB3 receptor transphosphorylation. The completion of these experiments will represent the effective execution of Specific Aim 3 and will result in the submission of a manuscript that is now in preparation.

III. Mechanism of Interaction between ErbB3 and Phosphatidylinositol 3-Kinase

Efforts in the previous funding period included the cloning and sequencing of the full-length rat ErbB3 cDNA. The results of this work are now published [(18), see Appendix]. Analysis of the deduced amino acid sequence of the C-terminal phosphorylation domain of the ErbB3 protein indicated the presence of 14 tyrosine residues in consensus sequences for the binding of known SH2 domain (src homology domain 2)-containing signalling molecules, such as Shc, Src and the p85 subunit of PI 3-kinase (19). Also identified in the C-terminal domain were two proline-rich sequences conforming to the consensus sequences for binding of known SH3 domain proteins, including the Src PTK and p85 (18). It was considered that the unique C-terminal domain of the ErbB3 protein might bind Src and p85, and possibly other previous uncharacterized SH2 and SH3 domain proteins. Indeed our previous work had shown the ability of ErbB3 to interact

with PI 3-kinase in cultured cells (20). To further explore the mechanism of interaction between ErbB3 and PI 3-kinase and to further address the goals of Specific Aim 4, we exploited the yeast two-hybrid system for the characterization of protein-protein interactions (21).

Use of the yeast two-hybrid system first required the construction of a Gal4 DNA binding domain (GBD) vector incorporating the C-terminal domain of the ErbB3 protein. Because it was envisioned that the interaction of p85 with the ErbB3 protein would require phosphorylation of the latter on tyrosine residues, the C-terminal ErbB3 domain in this construct was fused to the catalytic core of the epidermal growth factor (EGF) receptor PTK to construct a pGBT-EGFR/B3 hybrid cDNA to be used as "bait" with the yeast system (see Fig. 1). As a first test of this system, we expressed the p85 subunit of PI 3-kinase as a Gal4 transcriptional activation domain (GAD) fusion protein in the "prey" vector, pGAD-p85. If in the context of the GBT-EGFR/B3 fusion protein, the proper tyrosine residues of the ErbB3 C-terminus were phosphorylated by the EGF receptor PTK, then interactions between these phosphotyrosine residues and the SH2 domains of p85 could result in the reconstitution of a functional Gal4 transcription factor and the activation of β -galactosidase reporter gene expression in the yeast cells (21). Indeed the coexpression of the "bait" and "prey" vectors yielded high values of β -galactosidase activity in the yeast reporter strain (see Fig. 2), which indicated a high-affinity interaction between the ErbB3 C-terminus and the p85 protein.

A variety of structurally modified "bait" vectors were used in control experiments to demonstrate that the interaction between "bait" and "prey" vectors required the presence of the ErbB3 C-terminal phosphorylation domain (data not shown) and the intrinsic PTK activity of the EGF receptor PTK (see Fig. 2). Use of various modified "prey" constructs demonstrated that the interaction required the presence of SH2 domains in the p85 protein (Fig. 3). In summary, the hybrid GBT-EGFR/B3 "prey" protein was highly satisfactory for the investigation of interactions between the phosphorylated ErbB3 C-terminus and signal-transducing molecules such as p85, and yielded results relevant to the pursuit of Specific Aim 4. We are currently using this system to attempt to detect interactions between the ErbB3 protein and the Src PTK, a prototypical nonreceptor PTK containing SH2 and SH3 domains that has been predicted to interact with phosphorylated EGF receptor family members (22). The "bait" vector is also being used in library screens to identify and clone cDNAs encoding novel proteins that might interact with the activated ErbB3 protein.

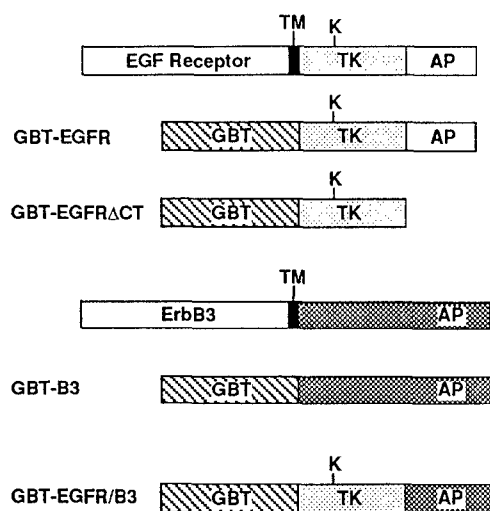


Fig. 1. Schematic structures of fusion protein constructs used as bait in yeast two-hybrid system assays. The full-length EGF and ErbB3 receptor proteins are shown with transmembrane (TM), protein tyrosine kinase (TK), and autophosphorylation (AP) domains indicated. The construct GBT-EGFR contains the TK domain of the EGF receptor fused to the DNA binding domain of the GAL4 transcription factor (GBT). GBT-EGFR Δ CT contains a C-terminally truncated EGF receptor TK domain lacking autophosphorylation sites. GBT-B3 contains the cytosolic domain of the ErbB3 protein, which is devoid of intrinsic protein kinase activity. GBT-EGFR/B3 is a chimeric construct composed of the EGFR TK domain fused to the autophosphorylation domain of the ErbB3 protein. A conserved lysine residue (K) in the EGF receptor TK domain essential for protein kinase activity is also indicated. This residue has been mutated to methionine in some constructs to abolish intrinsic kinase activity.

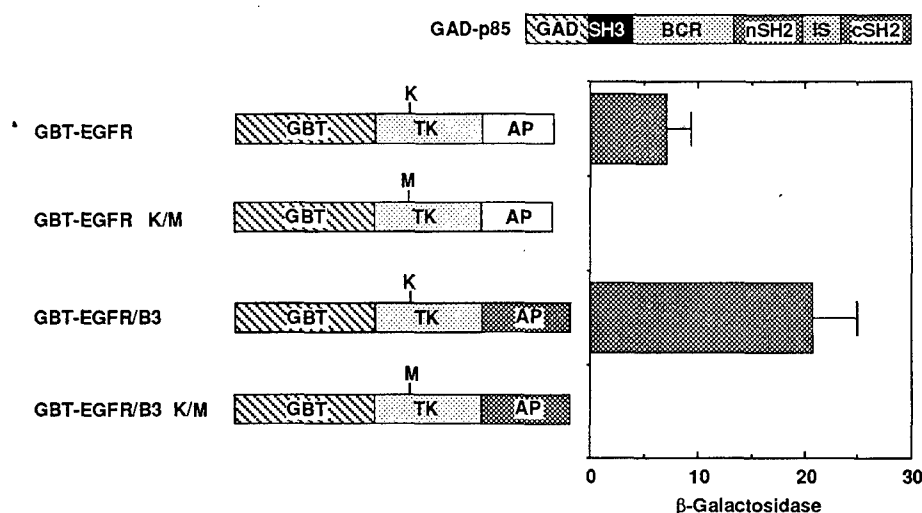


Fig. 2. Results of quantitative β -galactosidase activity assays demonstrating interactions between the EGF receptor and ErbB3 C-terminal domains with the p85 subunit of phosphatidylinositol 3-kinase. The kinase-inactivating amino acid substitution (K to M) in the EGF receptor PTK domain is shown to abolish high-affinity interactions.

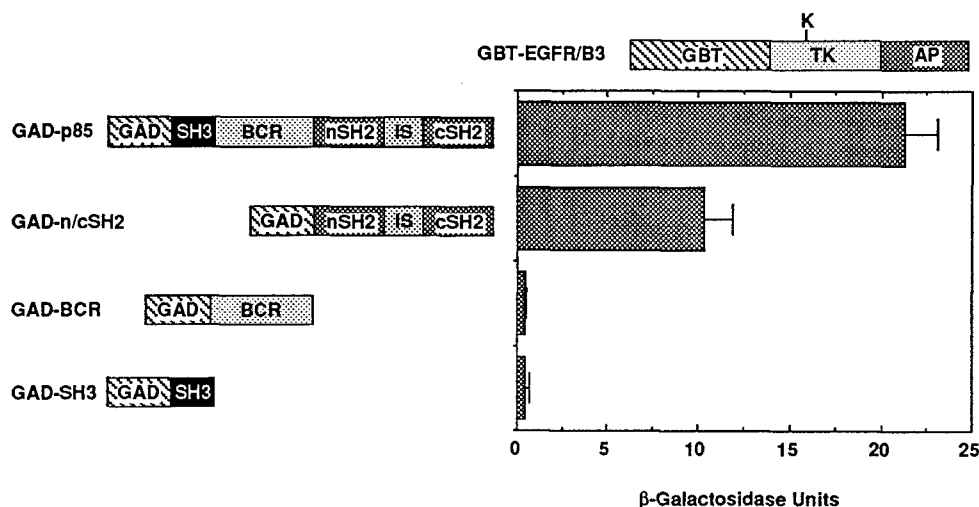


Fig. 3. SH2 domains of the p85 subunits of PI 3-kinase mediate interactions with the ErbB3 C-terminal domain. Gal4 transcriptional activation domain vectors expressing various segments of the p85 protein were tested for their ability to interact with the GBT-EGFR/B3 vector in the yeast two-hybrid system. The C-terminal domain containing two SH2 domains appeared to be largely responsible for mediating high-affinity interactions.

IV. Analysis of Phosphatidylinositol 3-Kinase Activation in Intact Breast Cancer Cells

An important goal of Specific Aim 4 is the demonstration of the heregulin- and ErbB2/ErbB3 coreceptor-dependent activation of PI 3-kinase in intact cultured cells. Hence, while a variety of evidence indicates that the PI 3-kinase enzyme interacts with the activated heregulin receptor complex, it is not clear whether this interaction results in significant alterations in the levels of various PI metabolites, specifically PI-(3,4)P₂ and PI-(3,4,5)P₃. As these PI metabolites function as intracellular second messenger molecules, determining how their intracellular levels change in response to heregulin will be crucial to understanding the ErbB2/ErbB3 signal transduction mechanism. In the past year we have begun to develop the technology necessary for the analysis of cellular phosphoinositides.

The standard analysis of PI 3-kinase reaction products, PI-3P, PI-(3,4)P₂ and PI-(3,4,5)P₃, is performed as follows (23). (1) Cultured cells are metabolically labeled with ³H-myo-inositol. (2) Cells are challenged with polypeptide growth factor (or vehicle in control experiments). (3) Lipids are extracted and subjected to deacylation. (4) Tritiated inositol lipid head groups are resolved and quantified by HPLC analysis with scintillation counting of fractions. The HPLC analysis requires the previous preparation of ³²P-labeled PI-3P, PI-(3,4)P₂ and PI-(3,4,5)P₃ standards. These standards are generated by incubating their precursors PI, PI-4P and PI-(4,5)P₂ with PI 3-kinase and [³²P]ATP. PI 3-kinase is isolated by immunoprecipitating cell lysates with a p85 regulatory subunit antibody.

We have only recently become proficient in each aspect of the PIP analysis. Initially, we experienced some difficulty in the generation of the PI-(3,4,5)P₃ standard via the phosphorylation of PI-(4,5)P₂, as it is in our hands generated with a much lower efficiency than are the other two standards. Success here was achieved by using larger quantities of immunoprecipitated PI 3-kinase, and by the inclusion of phosphatidylserine in the reaction medium. We have been able to routinely label cellular phosphoinositides, extract and deacylate these labeled lipids, and execute HPLC analysis. Presently we are performing control experiments in which the PIPs from NIH-3T3 fibroblasts treated with or without platelet-derived growth factor (PDGF) are analyzed. The synthesis of PI-3P, PI-(3,4)P₂ and PI-(3,4,5)P₃ in response to PDGF has been relatively well characterized (24,25). Assuming we are able to quantify the changes in PI 3-kinase products occurring in response to PDGF, we will then be able to analyze the heregulin-

dependent changes in PI metabolite levels in human breast cancer cell lines and in cell lines transfected with recombinant ErbB2 and ErbB3 proteins.

CONCLUSIONS

Studies completed in the second year of research have verified that the ErbB3 protein is devoid of intrinsic protein tyrosine kinase (PTK) activity and must function in cooperation with other ErbB family members that do possess PTK activity. Particularly illuminating, was our demonstration that the function of the ErbB2/ErbB3 coreceptor complex is completely abolished when the ErbB2 catalytic site is mutated by site-directed mutagenesis. A similar mutation of the PTK homology domain of the ErbB3 protein had negligible effects on coreceptor signalling. These findings highlight the significance of the coincident over-expression of multiple ErbB family members that has been frequently observed in breast cancer cells. Our investigations of the interaction of the phosphorylated ErbB3 C-terminus with signalling molecules, specifically the p85 subunit of phosphatidylinositol (PI) 3-kinase, again demonstrate the importance of ErbB2/ErbB3 transphosphorylation in coreceptor signalling. Continued efforts with the yeast two-hybrid system should allow a thorough dissection of the mechanism of interaction between the ErbB3 protein and its signalling targets, and will possibly also lead to the identification of novel ErbB3 target proteins.

Knowledge of the mechanism of ErbB2/ErbB3 coreceptor signalling is crucial to our understanding of the control of breast cancer cell proliferation, as many of the cultured breast cancer cells examined to date express abnormally high levels of both of these receptor proteins. The heregulin polypeptide, which is an activating ligand for the ErbB2/ErbB3 coreceptor, is known to be expressed and secreted by breast cancer cells, and could therefore be critical to the proliferation of those breast cancer cells expressing the coreceptor. Our efforts to isolate the heregulin gene promoter could lead to the identification of factors that regulate heregulin production and therefore preface the development of novel therapeutic strategies for the attenuation of breast cancer cell proliferation.

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APPENDICES

Manuscripts published or submitted in the funding period:

The first manuscript listed below was generated in the first and published in the second year of research. The second manuscript resulted from studies performed in the second year of research. Copies of each are appended to the progress report.

1. Hellyer, N.J., Kim, H.-H., Greaves, C.H., Sierke, S.L., and Koland, J.G. (1996) "Cloning of the Rat *ErbB3* cDNA and Characterization of the Recombinant Protein," *Gene* (in press).
2. Sierke, S.L., Cheng, K., Kim, H.-H., and Koland, J.G. (1996) "Biochemical Characterization of the Protein Tyrosine Kinase Homology Domain of the ErbB3 (HER3) Receptor Protein," *Biochemical J.* (accepted pending revision).

Abstracts presented in the funding period:

1. Shearer, M.C., Kim, H.-H., Kratz, D.A., and Koland, J.G. (1996) "Heregulin-dependent Activation of MAP Kinases in Cultured Rat Astrocytes," ASBMB, New Orleans, June
2. Hellyer, N.J., and Koland, J.G. (1996) "Characterizing the Interaction between ErbB3/HER3 and the p85 Subunit of Phosphatidylinositol 3-Kinase with the Yeast Two-Hybrid System," ASBMB, New Orleans, June

Personnel receiving pay from the negotiated effort:

1. John G. Koland, Ph.D. Principal Investigator
2. Deborah Kratz, B.S. Research Assistant II
3. Dolores Bravo, M.S. Research Assistant II

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Cloning of the rat *ErbB3* cDNA and characterization of the recombinant protein

(HER3; protein tyrosine kinase; Src homology domain; heregulin; oncogene)

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SUMMARY

Three cDNA fragments that encoded all but the extreme N terminus of the rat ErbB3 protein were cloned by low-stringency screening of a rat liver cDNA library with a human *ERBB3* probe. The remaining 5'-end of the cDNA was generated by a reverse transcription-polymerase chain reaction method, and a single full-length rat *ErbB3* cDNA was assembled. A comparison of the deduced amino acid (aa) sequences of human and rat ErbB3 was made, and the effects of certain aa substitutions in the putative protein tyrosine kinase domain were considered. The rat *ErbB3* cDNA was subsequently expressed in cultured NIH-3T3 mouse fibroblasts, in which a high level of approx. 180-kDa recombinant ErbB3 (re-ErbB3) was generated. The rat re-ErbB3 produced in transfected fibroblasts was responsive to the polypeptide, heregulin, a known ligand for ErbB3. Challenge of transfected fibroblasts with heregulin stimulated the phosphorylation of rat re-ErbB3 on Tyr residues and promoted its association with the p85 subunit of phosphatidylinositol 3-kinase. Together, these results indicate that a fully functional rat *ErbB3* cDNA has been isolated, and that fibroblast cells expressing this cDNA will be suitable for investigations of the signal transduction mechanism of ErbB3.

INTRODUCTION

The *ERBB* (*HER*) family of human genes has four identified members. The prototypical c-*ERBB* (*HER*)

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); EGF, epidermal growth factor; *ERBB3*/*ErbB3*, human/rat gene (DNA) encoding ErbB3 (also designated *HER3*); GRB2, growth factor receptor-bound protein 2; HRG, heregulin (Neu differentiation factor); oligo, oligodeoxynucleotide; PCR, polymerase chain reaction; PI, phosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PTK, protein tyrosine kinase(s); re-, recombinant; RT, reverse transcription(ase); SDS, sodium dodecyl sulfate; SH, Src homology domain; SHC, Src homology-2 α -collagen related protein; SLIC, single-strand ligation to ss-cDNA; ss, single strand(ed); STP, signal-transducing protein(s); TM, transmembrane; Xaa, any aa.

gene encodes the well studied epidermal growth factor (EGF) receptor and was first identified as the cellular homolog of the v-*ErbB* oncogene of avian erythroblastosis virus (Downward et al., 1984). The EGF receptor consists of a single polypeptide chain that forms an extracellular growth factor binding domain, a short transmembrane (TM) domain, and an intracellular protein tyrosine kinase (PTK) domain. The *ERBB2* (*HER2*) gene, first characterized as a homolog of the rat *Neu* oncogene, also encodes a polypeptide growth factor receptor with PTK activity (Stern et al., 1986). The *ERBB3* (*HER3*) (Kraus et al., 1989; Plowman et al., 1990) and *ERBB4* (*HER4*) (Plowman et al., 1993a) genes have been more recently identified by molecular cloning. The predicted amino acid (aa) sequences of human ErbB3 and ErbB4 are closely related to those of the EGF receptor and ErbB2, which suggests that these two proteins are also receptors with intrinsic PTK activity.

A number of distinct polypeptide ligands that bind and activate ErbB family receptor proteins have now been identified. However, the relationships between the individual ligands and the four ErbB proteins are complex and not fully resolved. Whereas EGF and transforming growth factor α (TGF α) are identified ligands for the EGF receptor (Gill et al., 1987), a variety of polypeptides in the heregulin (HRG) family, which includes the heregulins (Holmes et al., 1992), Neu differentiation factor (NDF) (Wen et al., 1992), and the glial growth factors (Marchionni et al., 1993), have been characterized as ligands for ErbB3. Recent evidence indicates that the high-affinity HRG receptor is actually an ErbB2/ErbB3 heterodimer, with the ErbB3 protein being itself a low-affinity HRG receptor (Sliwkowski et al., 1994; Tzahar et al., 1994). In addition, the ErbB4 protein has been demonstrated to be a high-affinity receptor for HRG (Plowman et al., 1993b; Tzahar et al., 1994).

The intrinsic PTK activity of the ErbB proteins has been generally considered to be essential for their functions in the activation of mitogenic and developmental signal transduction pathways (Schlessinger and Ullrich, 1992). Although the predicted PTK domains of the ErbB proteins have aa sequences that agree well with the consensus sequence for identified PTK (Hanks and Quinn, 1991), human ErbB3 is unique in that three of the aa residues invariantly conserved in PTK are found altered in its predicted PTK domain sequence (Kraus et al., 1989; Plowman et al., 1990). This suggests that ErbB3 might not possess intrinsic PTK activity, and could have a cellular function quite distinct from those of the other ErbB family receptor proteins. To this end, several attempts have been made to demonstrate an intrinsic PTK activity in the *ERBB3* gene product.

Kraus et al. (1993) have documented the constitutive phosphorylation of ErbB3 that is endogenously produced in high levels in certain cultured human breast cancer cell lines. A ligand-dependent activation of the ErbB3 PTK domain in the context of a recombinant chimeric protein that incorporates the extracellular and membrane-spanning domains of the EGF receptor has also been demonstrated (Kraus et al., 1993; Prigent and Gullick, 1994). In contrast, our laboratory (H.-H. K., S.L.S. and J.G.K., data not shown) and others (Guy et al., 1994) have failed to detect intrinsic PTK activity in recombinant and native ErbB3 proteins. To enable the further examination of the catalytic and signal-transducing potentials of ErbB3, we have now isolated a full-length rat *ErbB3* cDNA and generated the corresponding protein in a cultured cell system by gene transfection. In this paper, we present an analysis of this full-length rat *ErbB3* coding sequence and a preliminary characterization of the rat re-ErbB3 protein.

MRATGTLQVLCFLSLARGSEMGNQAVCPGTLNGLSVTDADNQYQTLKLYEKCEVVM	60
NJA GL F V E R	
GNLEIVLTGHNADLSFLQWIREVTAYVLVAMNEFSVLPLNLRVVRGTQVYDGKFAIFVM	120
G T	
LNNTNSSHARLQKFTQLTEILSGGVYIEKNDKLCMDITDWRDIVRVRGAEIVVKNG	180
RL D D	
ANCPPECHEVCKGRGPGPDDCQILTKTICAPQCNCRGFCFNPNCCHDECAGGCGSPQD	240
RS SE T H	
TDCFACRRFNSGACVPRCPPELVYNKLTFLQLEPNPHTKYQYGGVCVASCSPHNFVVDQTF	300
H Q	
CVRACPPDKMEVDKHLKMCPECGGLCPKACEGTGSGSRQYTDSSNIDGFVNCTKILGN	360
N F	
LDLITGLTGDPHKIPALDPEKLNVPRTVREITGYLNSWPPHMHNFVSFNLTTIGG	420
N	
RSLYNRGFSLLIMKNLNVTSLGFRSLKEISAGRVYISANQCLYHHSINWTRLLRGPSSE	480
I R KV T	
RLDIKYDRPLGECLAEGKVCDCPLCSGGCGWGPAGQCLSCRNYSREGVCVTNCFNGEP	540
HN RRD V SE T G H N	
REFVHEAQCFSCHEPLMEGTSTYNGSGSDACARCAHFRDGPCHVCNSCPHGLGAKGPI	600
A E Q A C T Q S V	
YKYPDAQNECRPCHENCTQGCNGPELQDCLGQAEVLMSKPHLVIAVTV--GLAVILMILG	658
V K TL IG T TM L IA V F M	
GSFLYWRGRRIQNKRAMRRYLGERGSEIEPLDPEKANKVLAIRFKETELRKLKVLGSGVF	718
T	
GTVHKGIWIPEGESIKIPVCIKVIDKSGRQSFQAVTDHMLAVSLDHAHIVRLGLGCPG	778
V A E I	
SSLQVLTQVLPGLSLDHDVQKQRETLGQQLLNWGVQIAKGYMYLEHSMVHRDLALRN	838
R GA N A	
MLKSPSQVQVADFGVADLLPDDKQLLHSEAKTPIKWMALESIHFGKYTHQSDVWSYGV	898
L Y	
VWELMTFGAEPIAGRLAEIPDLLEKGERLAQPOICTIDVYVMVKWCMIDENIRPTFKE	958
V	
LANEFTRMARDPPRYLVIKRASGPGTPPAEPVLTTELQEALEPELDLDLEAEAE	1018
E IA GP HG N K E V D	
GLA-TSLGSALSPLTGTTLTRFRGSQSLSPSSGYMFMNQSSLGAECLDSAVLGGREQFSK	1077
N T T V N GN S QE S SS RCP	
PISLHPPIPRGRPASSESEGHVTGSEAEQLQEKVSVCRSRSPRPRGDSAYHSQRHSL	1137
V M CL M	
TPVTPLSPPGLEEDNGYVMPDTHLRGASSSREGTLSSVGLSSVLGTEEEDEDEEYEM	1197
V K TP	
NRKRGSPPRPPEPSELEELGYEYMDVSGDLSASLGSTQSCPLHMAIVESAGTTPDEY	1257
R H H S VP M T	
EYNNRRRGAGGAGGDYAAAGACPAAEQGYEEMRAFGQPGHAPHVRYARKTLRSLEATD	1317
Q DG P S Q H	
SAFDNPDYWHSLRFPKANAQRT 1339	

Fig. 1. Deduced aa sequence of rat ErbB3. The rat *ErbB3* cDNA sequence encoded a protein of 1339 aa. Deviations in the human sequence (Kraus et al., 1989; Plowman et al., 1990) are listed below the rat sequence. The putative leader peptide and TM domain are indicated with bold underlines. The symbol (●) indicates three aa conserved in all characterized PTK that are substituted in the human ErbB3 sequence. Other identified sequence elements including several candidate binding sites for STP are indicated by underlining (see Table 1). The cDNA sequence has been deposited in GenBank/EMBL under accession No. U29339. **Methods:** A partial cDNA clone was isolated by screening a rat liver λ ZAPII library (Stratagene, La Jolla, CA, USA) at reduced stringency with a human *ERBB3* cDNA probe. Overlapping cDNAs were isolated by screening the same library at high stringency with restriction fragment probes derived from successively isolated rat *ErbB3* cDNAs. The remaining 5'-sequence of rat *ErbB3* cDNA was amplified by the 5'-SLIC/RT-PCR method (Dumas et al., 1991). First-strand cDNA synthesis was carried out with rat liver mRNA (1 μ g) (Clontech, Palo Alto, CA, USA), Superscript RT (GIBCO-BRL, Grand Island, NY, USA) (10 units/ μ l), and random hexamer oligo primers (3.3 ng/ μ l). Four independent PCR products were cloned and sequenced. One product representing the consensus sequence was subcloned with the overlapping cDNA fragments to yield a full-length rat ErbB3 coding sequence.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the rat *ErbB3* cDNA

Screening of a rat liver bacteriophage λ cDNA library with a human *ERBB3* cDNA probe yielded several cDNA fragments. Sequence analyses indicated that four of these cDNA fragments (1) each had high similarity to

the human *ERBB3* cDNA, (2) were overlapping, and (3) were derived from a single coding sequence, presumably that of rat *ErbB3*. However, the extreme 5'-end of the rat *ErbB3* message was not represented in these isolated cDNAs. The remaining 5'-end of the rat *ErbB3* cDNA was therefore generated by a RT-PCR method (see Fig. 1).

(b) Deduced aa sequence of rat *ErbB3*

The 5'-cDNA generated by RT-PCR and three cDNA fragments isolated from the cDNA library were together subcloned to yield a full-length rat *ErbB3* cDNA. The deduced aa sequence of rat *ErbB3* and the sequence deviations between human and rat *ErbB3* are shown in Fig. 1. Overall there was a 90% aa identity between the human and rat sequences. Two short gaps in the sequence alignment indicated the deletion in the rat sequence of two aa in the presumed TM domain and one aa in the C-terminal autophosphorylation domain. The sequence encoding the putative PTK domain of the rat protein showed a 96% identity with that of the human protein. The rat *ErbB3* gene product is predicted to consist of 1339 aa (147 578 Da).

Comparison of the deduced rat *ErbB3* aa sequence with the consensus sequence of the catalytic domains of known PTK (Hanks and Quinn, 1991) showed two substitutions of invariantly conserved aa (see Fig. 1). The residues Cys⁷³⁸ and His⁷⁵⁷ of the rat protein, which correspond respectively to Ala⁷¹⁹ and Glu⁷³⁸ of the human epidermal growth factor (EGF) receptor, deviate from the canonical sequence. Notably, Asp⁸³² of the rat *ErbB3* sequence, which corresponds to Asp⁸¹³ in the EGF receptor sequence, does agree with the consensus sequence, although it is found substituted in the human *ErbB3* sequence by Asn⁸³⁴. Given that the mutation of this conserved aspartate residue has been shown to abolish the PTK activity of the EGF receptor (Coker et al., 1994), the lack of this substitution in the rat protein could conceivably alter the catalytic properties of rat *ErbB3* relative to those of the human protein. Recently, Prigent and Gullick (1994) employed site-directed mutagenesis to explore the consequences of the substitution in human *ErbB3* of His⁷⁵⁹ and Asn⁸³⁴ for the normally conserved Glu and Asp residues. The low levels of PTK activity exhibited by wild-type *ErbB3* in immune-complex kinase assays were not found to be enhanced by a His⁷⁵⁹→Glu and Asn⁸³⁴→Asp double aa substitution.

Other notable structural features are found in the C-terminal domain of rat *ErbB3*. Of particular interest is the presence of several candidate Tyr residue phosphorylation sites in specific sequence motifs that constitute the recognition sites for SH2 domain (second domain of Src homology) proteins (Cohen et al., 1995; Pawson, 1995). The SH2 domains found in various signal-transducing

proteins (STP), such as PI 3-kinase, phospholipase C- γ 1, GRB2 and SHC, have been shown to bind to activated growth factor receptors and related proteins that contain phosphorylated Tyr residues. It has become apparent that each distinct SH2 domain protein recognizes phospho-Tyr residues in a specific sequence context (Songyang et al., 1993). The sequence motif Tyr-Xaa-Xaa-Met, the consensus binding site sequence for PI 3-kinase, is found repeated seven times in the C-terminal domain of human *ErbB3*, and the seven repeats of this sequence are found intact in the C terminus of the deduced rat *ErbB3* aa sequence (see Table 1). Two consensus binding sites for the GRB2 protein, Tyr-Met-Asn, are also found in the C-termini of both rat and human *ErbB3*. Also of interest is the overlapping of PI 3-kinase binding sites with these two GRB2 binding sites in the sequence element Tyr-Glu-Tyr-Met-Asn. Conceivably, these dual-specificity binding sites, depending upon which of the two Tyr residues were phosphorylated, could bind either PI 3-kinase or the GRB2 protein. A potential SH2 domain binding site for Src family PTK, Tyr-Glu-Glu-Met, is also present in both rat and human *ErbB3* sequences. Another motif observed in both the rat and human sequences is Tyr-Val-Met-Pro, which if phosphorylated could constitute a binding site for the SH2 domain-containing protein tyrosine phosphatase SH-PTP2. Finally, the binding site for SHC previously identified in human *ErbB3* (Prigent and Gullick, 1994) is again found in the rat sequence. The observed cross-species conservation of the aa sequences of these various SH2 domain binding sites is consistent with the assumption that *ErbB3* interacts functionally with multiple STP.

Recently it has been determined that the SH3 domains found in various STP bind specifically to certain Pro-rich sequences (Kapeller et al., 1994; Ren et al., 1993). Two such sequences, Pro-Arg-Pro-Pro-Arg-Pro and Pro-Lys-Pro-Pro-Lys-Pro, found in the p85 subunit of PI 3-kinase are part of the identified binding sites for the SH3 domains of Src family PTK (Kapeller et al., 1994). Also, the sequence Pro-Xaa-Xaa-Pro-Pro-Pro-Xaa-Xaa-Pro as found twice in the 3BP-1 protein has been considered a consensus SH3 domain binding site (Ren et al., 1993). The first element, Pro-Arg-Pro-Pro-Arg-Pro, is found within the C terminus of rat *ErbB3*, with the corresponding sequence in the human protein being Pro-His-Pro-Pro-Arg-Pro (see Table 1). A sequence very similar to the second element, Pro-Leu-His-Pro-Val-Pro-Ile-Met-Pro, is found in the human *ErbB3* C terminus, although the corresponding rat sequence, Pro-Leu-His-Pro-Met-Ala-Ile-Val-Pro, is less similar. Presumably these proline-rich sequences of rat *ErbB3* could interact directly with SH3 domain proteins, and perhaps in cooperation with the identified SH2 domain binding motifs could mediate high-affinity interactions with proteins

TABLE I

Potential binding sites for SH2 and SH3 domain proteins identified in human and rat ErbB3 aa sequences

SH2/SH3 domain protein ^a	Binding site ^b	Human ErbB3 ^c	Rat ErbB3
p85 (PI 3-kinase) (SH2)	Y (M/X)XM	Y ⁹⁴¹ MVM Y ¹⁰⁵⁴ MPM Y ¹¹⁹⁷ EYMN Y ¹²²² EYM Y ¹²⁶⁰ EYMN Y ¹²⁷⁶ AAM Y ¹²⁸⁹ EEM	Y ⁹³⁹ MVM Y ¹⁰⁵¹ MPM Y ¹¹⁹⁴ EYMN Y ¹²¹⁹ EYM Y ¹²⁵⁷ EYMN Y ¹²⁷³ AAM Y ¹²⁸⁶ EEM
GRB2/Sem-5 (SH2)	YMN	YEY ¹¹⁹⁹ MN YEY ¹²⁶² MN	YEY ¹¹⁹⁶ MN YEY ¹²⁵⁹ MN
SHC (SH2)	NPXY ^d	NPDY ^{1328e}	NPDY ¹³²⁵
SH-PTP2 (SH2)	YVXP	Y ¹¹⁵⁹ VMP	Y ¹¹⁵⁶ VMP
Src family kinases (SH2)	YEEM	Y ¹²⁸⁹ EEM	Y ¹²⁸⁶ EEM
p85 (SH3)	P(R/K)PP(R/K)P ^f	P ¹²⁰⁶ HPPRP	P ¹²⁰⁹ RPPRP

^a Specific proteins containing SH2 or SH3 domains (second or third domain of Src homology, respectively) as indicated in parentheses.^b Consensus SH2 domain binding motifs selected from random peptide libraries with specific SH2 domain probes (Songyang et al., 1993). Phosphorylated Tyr residues (Y) in binding site sequences are indicated in bold.^c Sites identified in the published aa sequence of human ErbB3 (Kraus et al., 1989; Plowman et al., 1990) with numbering according to Kraus et al. (1989).^d Consensus SHC binding site as previously described (Prigent and Gullick, 1994).^e Identified SHC binding site in human ErbB3 (Prigent and Gullick, 1994).^f From two identified Pro-rich SH3 domain binding site sequences in the p85 subunit of PI 3-kinase (Kapeller et al., 1994).

containing both SH2 and SH3 domains, such as GRB2, p85 and Src family PTK.

(c) Cellular expression of the isolated *ErbB3* cDNA

In order to verify that the full-length cDNA generated by ligation of the several isolated cDNA molecules encoded a protein consistent with the predicted structure of ErbB3, this cDNA was subcloned into a mammalian cell expression vector and transfected into cultured mouse NIH-3T3 fibroblasts. Lysates of the transiently transfected fibroblasts were analyzed by SDS-PAGE and immunoblotting with an ErbB3-specific Ab. A relatively high level of approx. 180-kDa immunoreactive protein was detected in lysates of fibroblasts transfected with the rat *ErbB3* expression vector under conditions of optimal pH (see Fig. 2). Although the molecular mass of recombinant ErbB3 (re-ErbB3) indicated by SDS-PAGE was significantly greater than that predicted by the cDNA sequence, it was similar to that of endogenous ErbB3 of human breast cancer cells (data not shown). Apparently both the rat and human ErbB3 proteins are subject to glycosylation.

(d) Activation of rat re-ErbB3 by the HRG-β peptide

The EGF-related polypeptide HRG-β1, previously characterized as an activating ligand for human ErbB3 (Carraway et al., 1994; Sliwkowski et al., 1994), was tested as an activator of rat re-ErbB3 in transfected NIH-3T3 fibroblasts. While a constitutive phosphorylation of re-ErbB3 was detected by immunoprecipitation with an

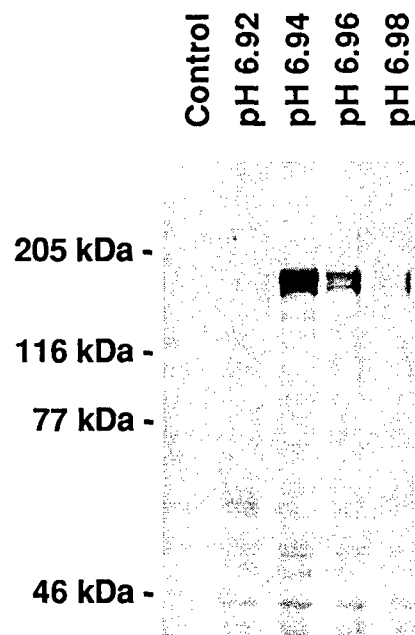


Fig. 2. Transient expression of the rat *ErbB3* cDNA in mouse NIH-3T3 fibroblasts. Subconfluent NIH-3T3 cells in 75-cm² flasks were transfected with 20 µg of pcDNA3-*ErbB3* plasmid per flask by a modified Ca-phosphate method (Sambrook et al., 1989). Buffers of slightly varying pH were used to optimize transfection efficiency. At 48 h post-transfection cells were detergent-lysed and samples of the lysates subjected to 0.1% SDS-7% PAGE and immunoblotting with ErbB3-specific monoclonal Ab 2F12 (Kim et al., 1994) (NeoMarkers, Fremont, CA, USA). Immunoblots were visualized with the ECL detection system (Amersham, Arlington Heights, IL, USA). Parental NIH-3T3 cells were analyzed for comparison (Control).

ErbB3-specific Ab and immunoblotting with antiphospho-Tyr, challenge of the transfected fibroblasts with HRG- β 1 clearly enhanced the phosphorylation of re-ErbB3 (see Fig. 3). Quantitation of immunoblots by densitometry yielded the average fold-stimulation of re-ErbB3 phosphorylation as 3.3 ± 0.9 ($n=4$). Considering that previous studies have demonstrated that ErbB2 and ErbB3 function as coreceptors for the HRG polypeptide (Sliwkowski et al., 1994) and that ErbB4 is also responsive to HRG (Plowman et al., 1993b), it is possible that an endogenous ErbB family member present at low levels in the transfected NIH-3T3 fibroblasts was involved in the observed stimulation of ErbB3 phosphorylation by HRG. As we have in numerous attempts failed to detect intrinsic PTK activity in the rat *ErbB3* gene product (data not shown), we assume that

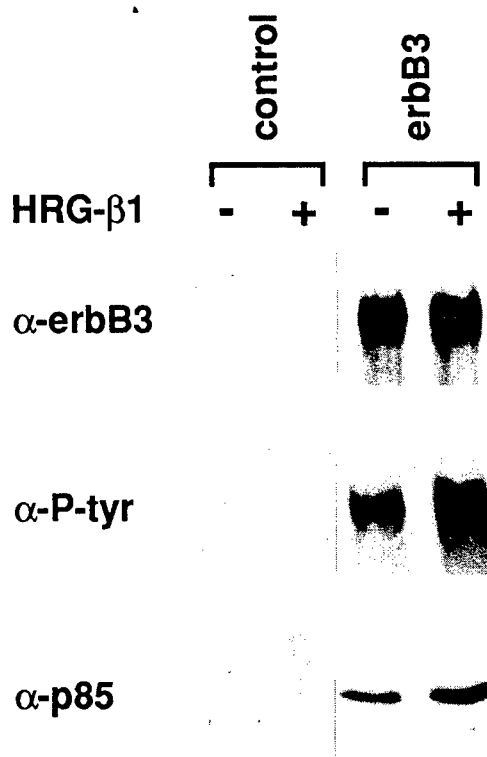


Fig. 3. Activation of rat re-ErbB3 protein by HRG- β 1. Mouse NIH-3T3 fibroblasts were transfected with the pcDNA3-*ErbB3* expression vector (see Fig. 2) and cultured under neomycin (G418) selection. Cells stably expressing the rat *ErbB3* cDNA were serum-starved for 16 h and then incubated for 5 min at 37°C in the presence or absence of an HRG- β 1 re-peptide fragment (H.-H. K., J.G.K., and S. Campion, data not shown) incorporating aa 177 to 241 of the HRG- β 1 precursor molecule (Holmes et al., 1992) at a concentration of 50 nM. Control cells transfected with the parent pcDNA3 expression vector (Invitrogen, San Diego, CA, USA) were similarly treated. Cell lysates were immunoprecipitated with ErbB3-specific monoclonal Ab 2F12, and immunoprecipitates were subjected to 0.1% SDS-7%-PAGE and immunoblotting with either Ab 2F12 (α -ErbB3), antiphospho-Tyr Ab PY20 (α -P-tyr) (Leinco Technologies, St. Louis, MO, USA), or a polyclonal Ab recognizing the p85 subunit of PI 3-kinase (α -p85) (Transduction Laboratories, Lexington, KY, USA) as indicated.

a distinct PTK(s) was responsible for both the constitutive and HRG-stimulated phosphorylation of re-ErbB3 in the transfected fibroblasts. Notably, we and others have demonstrated an EGF-dependent phosphorylation of ErbB3 in cultured cells containing both the EGF receptor and ErbB3 (Kim et al., 1994; Soltoff et al., 1994).

As previously documented by studies of human ErbB3 (Fedi et al., 1994; Kim et al., 1994; Soltoff et al., 1994), the p85 subunit of PI 3-kinase was found to immunoprecipitate with phosphorylated rat re-ErbB3 (see Fig. 3). Exposure of the transfected fibroblasts to HRG- β 1 significantly increased the quantity of p85 detected in ErbB3 immunoprecipitates. The HRG-dependent binding of this STP protein was possibly mediated by the phosphorylation of one or more of the Tyr-Xaa-Xaa-Met p85-binding motifs present in the ErbB3 C terminus.

(e) Conclusions

(1) A rat liver cDNA encoding a 1339-aa protein with 90% sequence identity with human ErbB3 has been isolated. Presumably derived from the rat *ErbB3* gene, this cDNA was expressed in cultured mouse NIH-3T3 fibroblasts, in which a high level of the approx. 180-kDa rat re-ErbB3 protein could be generated.

(2) The ErbB3 protein isolated by immunoprecipitation of cell lysates was constitutively phosphorylated on Tyr residues. Prior challenge of the cells with HRG- β 1 led to an enhanced ErbB3 phosphorylation, which indicated that rat ErbB3 may be a functional HRG receptor.

(3) Immunoprecipitation experiments indicated that a fraction of the total p85 protein in the transfected fibroblasts was constitutively associated with rat ErbB3. Challenge of the transfected cells with HRG- β 1 augmented the quantity of ErbB3-associated p85. Together these results indicated that the isolated rat *ErbB3* cDNA encodes a functional receptor protein that is responsive to HRG and can transduce signals to the PI 3-kinase pathway.

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**Biochemical characterization of the protein tyrosine kinase
homology domain of the ErbB3 (HER3) receptor protein**

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The putative protein tyrosine kinase domain (TKD) of the ErbB3 (HER3) receptor protein was generated as a histidine-tagged recombinant protein (hisTKD-B3) and characterized enzymologically. Circular dichroism spectroscopy indicated that the hisTKD-B3 protein assumed a native conformation with a secondary structure similar to that of the epidermal growth factor (EGF) receptor TKD. However, when compared to the EGF receptor-derived protein, hisTKD-B3 exhibited negligible intrinsic protein tyrosine kinase activity. Immune complex kinase assays of full-length ErbB3 proteins also yielded no evidence of catalytic activity. A fluorescence assay previously used to characterize the nucleotide binding properties of the EGF receptor indicated that the ErbB3 protein was unable to bind nucleotide. The hisTKD-B3 protein was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase, which suggested that *in vivo* phosphorylation of ErbB3 in response to EGF could be attributed to a direct cross-phosphorylation by the EGF receptor protein tyrosine kinase.

INTRODUCTION

Discovered by molecular cloning [1,2], the *ErbB3* gene encodes a member of the ErbB subfamily of receptor protein tyrosine kinases [3]. Like the prototypical epidermal growth factor (EGF) receptor, the ErbB3 protein is predicted to consist of an extracellular ligand-binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain, and a C-terminal phosphorylation domain. Despite its structural similarity to other ErbB family receptors (EGF receptor, ErbB2/Neu, ErbB4), the presence of protein tyrosine kinase activity in ErbB3 has been questioned [2], as the deduced amino acid sequence of the protein shows three substitutions for residues invariantly conserved in all protein tyrosine kinases with known sequence [4]. Efforts to resolve this question have led to conflicting results. Two groups have detected ligand-stimulated protein tyrosine kinase activity in a chimeric EGF receptor/ErbB3 protein, and concluded that the ErbB3 cytosolic domain possesses intrinsic catalytic activity [5,6]. However, a third group found negligible protein kinase activity in a recombinant bovine ErbB3 protein [7].

Recently, the ErbB3 protein has been shown to bind EGF-related polypeptides in the neuregulin (heregulin) family [8-10]. In cultured cells expressing ErbB3, the protein has been seen to be phosphorylated on tyrosine residues in response to EGF or neuregulin [11-13]. As this phosphorylation is dependent upon the co-expression of either EGF receptor or ErbB2 [14-17], it has been considered that the ErbB3 protein may be a physiologic substrate for the protein tyrosine kinase activities of the EGF receptor and ErbB2. Indeed it appears that the ErbB3 protein may form receptor heterodimers with either the EGF receptor or ErbB2 protein (reviewed in [18-20]). The role of any intrinsic protein tyrosine kinase activity of

ErbB3 in the phosphorylation of ErbB3 and its associated ErbB family members within the context of receptor heterodimers remains unclear.

In order to assess the catalytic potential of ErbB3, the cytosolic domain of the protein and that of the well characterized EGF receptor were generated by use of the baculovirus/insect cell expression system. The purified recombinant proteins were characterized by circular dichroism spectroscopy, protein tyrosine kinase activity assays, and a recently described nucleotide binding assay [21]. The recombinant ErbB3 protein was seen to be devoid of intrinsic protein tyrosine kinase activity, and indeed appeared unable to bind nucleotide. The ErbB3 cytosolic domain was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase. Together these results indicated that the observed phosphorylation of ErbB3 in the cellular context might be effected by the protein tyrosine kinase activities of other ErbB family members.

EXPERIMENTAL PROCEDURES

Cell lines and reagents

All cell lines were purchased from American Type Culture Collection and cultured as recommended. 2'(3')-O-(2,4,6-trinitrophenyl)-adenosine 5'-triphosphate (TNP-ATP) was obtained from Molecular Probes, Inc. γ [³²P]ATP (~3,000 Ci/mmol) was supplied by Dupont-New England Nuclear. ErbB3-specific (2F12) and EGF receptor-specific (LA1) monoclonal antibodies were purchased from NeoMarkers and Upstate Biotechnology, respectively. Phosphotyrosine-specific monoclonal antibody (PY20) was obtained from Leinco Technologies. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The fusion protein GST-TK7 [22] and NIH-3T3 cells expressing the rat ErbB3 cDNA [23] have been previously described.

Expression of EGF receptor and ErbB3 protein tyrosine kinase domain forms

Recombinant human EGF receptor and rat ErbB3 protein tyrosine kinase domain (TKD) forms, each with a Met-His-His-His-His-His leader peptide, were expressed with the baculovirus/insect cell system. The hisTKD38 coding sequence was generated from the EGF receptor cDNA in pMMTV-ER [24] by PCR with the primers 5'-TGCTCTAGACCATGCACCACCACCACCACCACCGAAGGCGCC-ACATCGTTTCGG-3' (forward) and 5'-CCCCCGGGCTAGTTGGAGTCTGTAGG-ACTTGGCAA-3' (reverse). The forward primer included an XbaI restriction site, a start codon (underlined), and six His codons, as well as the coding sequence for amino acid residues 645-651 of the EGF receptor. The reverse primer was complementary to the coding sequence for residues 965-972 of the EGF receptor, and introduced a stop codon (underlined) and a SmaI restriction site into the PCR product. The resulting PCR product was subcloned into the baculovirus transfer

vector pAcYMP1 [25] to yield pAc-TKD38. A baculovirus transfer vector for hisTKD61 (pAc-TKD61) was generated by cloning a cDNA fragment encoding the EGF receptor C-terminus into pAc-TKD38.

The coding sequence for the rat ErbB3 TKD was amplified by PCR from a previously characterized rat ErbB3 cDNA clone, pBS-rB3 [23]. The forward primer, 5'-TGCTCTAGACCATGCACCACCACCACCACCACCGAATTCGGATTCAGAACAAAAGGGCTA-3', included an XbaI site, a start codon (underlined), six His codons, and the codons for amino acid residues 668-674 of ErbB3. The reverse primer, 5'-ACAAGCTGCAGAGATGAC-3', was complementary to a coding sequence within the rat ErbB3 cDNA downstream of a unique NdeI restriction site. The resulting PCR product was cloned into pBS-rB3 to yield a cDNA encoding the hisTKD-B3 protein, which was then subcloned into pAcYMP1. The authenticity of the PCR-amplified sequences present in each transfer vector was directly verified by DNA sequencing.

The purified baculovirus transfer vectors were cotransfected with BaculoGold baculovirus DNA (Pharmingen) into cultured Sf21 cells [26]. Recombinant baculovirus clones were isolated by an end point dilution method [27], and viral clones expressing high levels of the recombinant TKDs were identified by immunoblotting lysates of virally infected Sf21 cells.

For large-scale preparation of recombinant proteins, Sf21 cells were grown in spinner flask culture (125 ml) to a density of $1-2 \times 10^6$ cells/ml, then infected with recombinant virus (~ 10 PFU/cell) [26]. At 48 h post-infection, cells were harvested and washed gently in 20 ml of insect cell lysis buffer [20 mM Tris/HCl, 0.5 M sodium chloride, 5 mM imidazole, 1 μ g/ml pepstatin A, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9] supplemented with 250 mM sucrose. The cells were resuspended in 10 ml of sucrose-free lysis buffer and sonicated. The homogenate was clarified by

centrifugation for 20 min at 80,000 g, and supplemented with Triton X-100 to a final concentration of 0.05%. The solution was applied to a 5 ml iminodiacetic acid-Sepharose-6B column (Sigma) that had been charged with 50 mM nickel sulfate and equilibrated with binding buffer (20 mM Tris/HCl, 0.5 M sodium chloride, 0.05% Triton X-100, pH 7.9) supplemented with 5 mM imidazole. The column was washed with ten column volumes of binding buffer (5 mM imidazole) and six column volumes of binding buffer supplemented with 60 mM imidazole, then eluted with binding buffer supplemented with 250 mM imidazole. Peak fractions in the eluent were identified by protein assays [28] and pooled. Free imidazole was removed by extensive dialysis against TKD dialysis buffer [20 mM Tris/HCl, 100 mM sodium chloride, 10% (v/v) glycerol, 0.05% Triton X-100, pH 8.0]. The purified TKD forms (~95% pure, typically 1-2 mg total protein) were supplemented with dithiothreitol to 1 mM and glycerol to 45% (v/v) and stored at -20°C. All purification steps were done at 4°C or on ice. In protein purifications for circular dichroism measurements, Triton X-100 was omitted from the column elution and final dialysis buffers.

***In vitro* protein tyrosine kinase assays**

TKD proteins (0.25 μ M) were incubated for 5 min at room temperature in TKD dialysis buffer (36 μ l total volume) with 15 μ M [γ^{32} P]ATP (~10⁴ cpm/pmol), 10 mM MnCl₂ or MgCl₂, and 0.1% Triton X-100 added to the indicated concentrations, and with or without the substrate GST-TK7 (5 μ g) [22]. After quenching the reactions by the addition of SDS-PAGE sample buffer, phosphoproteins were resolved by SDS-PAGE [29] and detected by autoradiography.

The cross-phosphorylation of the ErbB3 TKD (hisTKD-B3) by the truncated EGF receptor TKD (hisTKD38) was assayed as described above, except that the incubations were done for 15 min at room temperature. Phosphoproteins were

then identified either by immunoblotting with the phosphotyrosine-specific antibody PY20 or by autoradiography. The kinetics of the cross-phosphorylation reaction were assayed by incubating hisTKD38 (0.25 μ M) in the presence of 10 mM MnCl_2 , 15 μ M [$\gamma^{32}\text{P}$]ATP, and varying concentrations of hisTKD-B3 (0-2.5 μ M) for 5 min at room temperature in TKD dialysis buffer. The final glycerol concentrations of the samples were adjusted to a constant 30% (v/v). The ^{32}P -labeled phosphoproteins were resolved by SDS-PAGE, identified by autoradiography, and quantified by scintillation counting of bands excised from dried gels. V_{max} and K_{M} were determined by the fitting of rate equations with a nonlinear least squares minimization algorithm [30], and the hyperbolic curve generated is shown in Figure 6B.

In immune complex kinase assays, immunoprecipitates were incubated with [$\gamma^{32}\text{P}$]ATP ($\sim 10^4$ cpm/pmol) for 10 min at room temperature. Reactions contained 40 mM Hepes/Na (pH 7.4), 0.05% Triton X-100, 10 mM MnCl_2 , or 10 mM MgCl_2 and 3 mM MnCl_2 , and 17 μ M ATP. 5xSDS-PAGE sample buffer was added to stop the reactions, and the samples were subjected to electrophoresis and autoradiography.

Fluorescence spectroscopic analysis of nucleotide binding

Binding of the TNP-ATP nucleotide analog to the recombinant TKDs was analyzed by a recently described fluorescence assay [21]. Briefly, fixed concentrations of recombinant protein were titrated with increasing concentrations of TNP-ATP (0 - 7.5 μ M) as the fluorescence of the nucleotide was recorded. Fluorescence titration data were corrected for the contribution of both free and nonspecifically bound TNP-ATP, as determined by titrations performed with the inclusion of excess ATP, and for inner filter quenching effects observed at high TNP-ATP

concentrations. Dissociation constants for TNP-ATP binding were subsequently determined by fitting of a theoretical binding equation to the titration data [21].

Circular dichroism spectroscopic measurements

UV circular dichroism spectra of recombinant proteins were recorded with an Aviv 62DS instrument with solutions of 2 μ M protein in 10 mM Tris/HCl, 50 mM sodium chloride, 25% (v/v) glycerol, pH 7.9 held in 2-mm cells thermostated at 4°C. A solvent blank spectrum was subtracted from each protein spectrum. Analysis of circular dichroism spectra for determination of the content of secondary structural elements was done with the aid of spectral decomposition software [31].

RESULTS

Generation and characterization of recombinant EGF receptor and ErbB3 protein tyrosine kinase domains

In order to compare the catalytic properties of the EGF receptor and the ErbB3 protein, the protein tyrosine kinase domains (TKDs) of these receptors were expressed as recombinant proteins with the baculovirus/insect cell system. Baculovirus expression vectors for two distinct EGF receptor TKD forms, one with an authentic C-terminus (hisTKD61) and one with a highly truncated C-terminus (hisTKD38), and a full-length ErbB3 TKD form (hisTKD-B3) were constructed (Figure 1). The three recombinant TKDs were expressed in Sf21 cells, and each of these proteins was effectively purified by Ni²⁺-chelating column chromatography (Figure 2A).

The secondary structures of the purified recombinant proteins were analyzed by circular dichroism spectroscopy. The spectra of the C-terminally complete hisTKD61 and hisTKD-B3 proteins were qualitatively similar, and spectral decomposition analysis [31] indicated similar contents of α -helix, β -sheet, β -turn and random structural elements (Figure 2B). Given that the EGF receptor-derived hisTKD61 protein was found to possess a catalytic activity comparable to that of the native EGF receptor protein (data not shown), it was assumed that this recombinant protein was folded in a native conformation. The similarity of the circular dichroism spectrum of the hisTKD-B3 protein to that of the hisTKD61 protein then suggested that the ErbB3-derived protein also assumed a native conformation.

Catalytic activities of recombinant EGF receptor and ErbB3 TKDs

Previous studies of a full-length EGF receptor TKD expressed in the baculovirus/insect cell system indicated that the hisTKD61 protein would be an active protein tyrosine kinase showing selectivity for Mn^{2+} over Mg^{2+} as an activating metal ion [32,33]. The hisTKD38 protein was also expected to be fully active, although it was anticipated that this truncated protein would lack the strong autophosphorylation activity of the full-length TKD. The autophosphorylation and substrate phosphorylation activities of the two recombinant EGF receptor TKD forms were compared with those of the ErbB3-derived protein (Figure 3). These experiments employed a recombinant fusion protein (GST-TK7) known to be a substrate for the EGF receptor and c-Src protein tyrosine kinases [22], and both Mg^{2+} and Mn^{2+} were tested as activators of the phosphorylation reactions.

Whereas the hisTKD61 protein showed a strong autophosphorylation, autophosphorylation of the hisTKD38 and hisTKD-B3 proteins was much weaker. Phosphoamino acid analyses (data not shown) indicated that while the weak autophosphorylation of the hisTKD38 protein corresponded to the incorporation of phosphotyrosine, the hisTKD-B3 protein was not phosphorylated on tyrosine residues (see also Figure 6A). Each of the three TKD forms was phosphorylated to a very small extent on serine and threonine, which was apparently due to a contaminating serine/threonine kinase activity. Significantly, the substrate phosphorylation activity of the hisTKD-B3 protein was negligible compared to that of the EGF receptor-derived TKDs. Several other attempts to detect protein tyrosine kinase activity in the ErbB3 TKD also yielded negative results. For example, when a distinct ErbB3 TKD lacking the hexa-His leader peptide was

generated with a vaccinia virus expression system, intrinsic protein tyrosine kinase activity was again not evident (data not shown).

Catalytic activity of the full-length ErbB3 protein *in vitro*

The protein tyrosine kinase activity of the full-length ErbB3 protein was also assessed. Here, the native ErbB3 protein was immunoprecipitated from cells expressing the protein at a high level either as a consequence of gene transfection (3T3-B3 cells) or tumorigenesis (MDA-MB-453 and SK-BR-3) (Figure 4A). For comparison, the EGF receptor was immunoprecipitated from MDA-MB-468 cells. Immunoprecipitated proteins were incubated with [γ^{32} P]ATP and divalent metal ions. As expected, EGF receptor immune complexes showed strong autophosphorylation. In contrast, ErbB3 immunoprecipitates exhibited negligible autophosphorylation activity (Figure 4B). Neither varying the assay conditions nor stimulating with the ligand neuregulin led to the detection of ErbB3 kinase activity (data not shown). Both rat and human ErbB3 proteins were tested here, as the transfected NIH-3T3 cells expressed the rat ErbB3 protein and the cancer cell lines used were derived from human breast carcinomas.

Nucleotide binding properties of EGF receptor and ErbB3 TKD proteins

Previously, we have shown that the fluorescent nucleotide analog trinitrophenyl-ATP (TNP-ATP) binds to recombinant EGF receptor TKD forms, and that this binding can be conveniently monitored by measuring the enhancement of TNP-ATP fluorescence that occurs upon binding to the TKD [21]. TNP-ATP was also found to be a functional substrate for the EGF receptor protein tyrosine kinase, that apparently mimics the authentic substrate ATP. The TNP-ATP nucleotide binding exhibited by the EGF receptor-derived hisTKD61 protein was directly compared with that of the hisTKD-B3 protein (see Figure 5). Whereas the

hisTKD61 protein bound the nucleotide analog with a dissociation constant in the micromolar range ($K_d = 0.75 \pm 0.24 \mu\text{M}$), there was no detectable interaction of the nucleotide analog with the ErbB3-derived protein. Failure of the fluorescent nucleotide analog to interact with hisTKD-B3 precluded attempts to directly address the ATP binding properties of this protein. However, the inability of the ErbB3 protein to bind the nucleotide analog was certainly consistent with its observed lack of protein tyrosine kinase activity.

ErbB3 as a protein tyrosine kinase substrate

In our earlier work [34], C-terminal sequences of the ErbB3 receptor protein were found to be excellent substrates for the EGF receptor protein tyrosine kinase with K_M values ranging from 1 - 30 μM . This suggested that if the ErbB3 receptor was not itself an active protein kinase, it might serve as a substrate for another receptor protein kinase in the ErbB family. To examine the potential for EGF receptor/ErbB3 cross-phosphorylation, the EGF receptor-derived hisTKD38 protein was incubated with the ErbB3-derived hisTKD-B3 under phosphorylating conditions (Figure 6A). Whereas the hisTKD38 and hisTKD-B3 proteins alone showed negligible autophosphorylation activities when compared to the C-terminally complete hisTKD61 protein, hisTKD-B3 was strongly phosphorylated upon incubation with hisTKD38. This phosphorylation could be detected either by autoradiography of ^{32}P -labeled proteins, or by immunoblotting with antiphosphotyrosine (Figure 6A). The K_M and V_{max} for phosphorylation of the hisTKD-B3 substrate by the hisTKD38 protein kinase were approximately 0.5 μM and 1.4 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively (Figure 6B). Hence, the hisTKD-B3 protein exhibited a K_M value among the lowest documented for substrates for the EGF receptor protein tyrosine kinase.

DISCUSSION

The *ErbB3* gene product has been predicted to be a receptor protein tyrosine kinase similar in structure and function to other EGF receptor family members [1,2]. We have attempted to detect the intrinsic protein tyrosine kinase activity in the ErbB3 protein by various approaches. As the protein tyrosine kinase domains of a variety of other receptors have been produced in catalytically active form with the baculovirus system [25,35-38], we applied this system in the generation an ErbB3 cytosolic domain protein (hisTKD-B3). Circular dichroism spectroscopic measurements indicated that the hisTKD-B3 protein was folded in conformation similar to that of the corresponding EGF receptor cytosolic domain (hisTKD61), which displayed robust catalytic activity. However, the recombinant ErbB3 protein exhibited negligible catalytic activity under the same experimental conditions (Figure 3). Immune complex kinase assays of full-length ErbB3 proteins also failed to demonstrate intrinsic kinase activity (Figure 4). These results lead to the conclusion that the ErbB3 protein is not intrinsically a protein kinase.

The potential of the ErbB3 protein to bind nucleotide substrates was assessed with the aid of the fluorescent nucleotide analog TNP-ATP, which has previously been used to characterize the nucleotide binding properties of the EGF receptor TKD [21]. Whereas the EGF receptor-derived hisTKD61 protein again bound TNP-ATP with high affinity (Figure 5), there was no observed enhancement of TNP-ATP fluorescence in the presence of the hisTKD-B3 protein. Because there was no apparent interaction of the analog with hisTKD-B3, it was not possible to use this assay to investigate the potential interaction of ErbB3 with the authentic substrate ATP. In a previous study of recombinant bovine ErbB3 [7], the receptor protein was seen to be specifically labeled by 5'-*p*-fluorosulfonylbenzoyladenosine,

although again no evidence for intrinsic kinase activity was obtained. Given that the ErbB3 cytosolic domain here did not interact with the analog TNP-ATP and also showed no catalytic activity, it is reasonable to suspect that the ErbB3 protein may be unable to bind ATP in the same manner as other active protein tyrosine kinases.

The apparent absence of catalytic activity and failure to bind nucleotide substrate might be explained by the occurrence of nonconservative amino acid substitutions in the putative protein tyrosine kinase domain of ErbB3. Specifically, the residues Cys-721, His-740 and Asn-815 in human ErbB3 [2] correspond to Ala, Glu and Asp, respectively, in all other known protein tyrosine kinases [4]. Sequencing of the rat ErbB3 cDNA has revealed an Asp residue corresponding to Asn-815 in human ErbB3 [23], which suggested that the rat ErbB3 protein, unlike human ErbB3, might possess kinase activity. However, neither rat nor human ErbB3 showed evidence of catalytic activity in this study (Figure 4).

While a previous study of the bovine ErbB3 protein also yielded no indication of significant kinase activity [7], an apparent intrinsic protein tyrosine kinase activity was detected in other investigations of the human ErbB3 protein [5,6]. In these latter studies, *in vitro* phosphorylation of ErbB3 in an immune complex [5] and EGF-stimulated *in vivo* phosphorylation of a chimeric receptor consisting of the extracellular domain of EGF receptor and cytosolic domain of ErbB3 were demonstrated [5,6]. It is possible that this observed ErbB3 phosphorylation resulted from the action of an associated non-ErbB3 kinase. For example, the *in vivo* and *in vitro* phosphorylations of kinase-deficient mutant forms of the EGF receptor have been demonstrated [39,40], and an ectopically expressed kinase-deficient EGF receptor mutant was shown to be cross-phosphorylated by endogenous wild-type EGF receptors [41].

The ErbB3 protein has been shown to function with ErbB2/neu as a high affinity coreceptor for the neuregulin (heregulin) peptide [8,10]. Also, the EGF-dependent phosphorylation of the ErbB3 protein in human cancer cells expressing high levels of both EGF receptor and ErbB3 has been documented [11,12]. A variety of recent evidence is consistent with a general model in which pairs of distinct ErbB family receptor proteins function as receptor heterodimers [19]. In this model, receptor heterodimerization provides a mechanism for diversifying the signal transduction pathways activated by polypeptide growth factors in the EGF family. As specific phosphorylated tyrosine residues within the unique C-termini of the ErbB family members have been shown to function as docking sites for distinct signal-transducing proteins such as phospholipase C, phosphatidylinositol 3-kinase, Grb2 and Shc [42], receptor phosphorylation in the context of heterodimers is a critical event in ErbB family receptor signal transduction. If devoid of intrinsic protein tyrosine kinase activity, the ErbB3 protein would be phosphorylated only in association with other ErbB family receptor proteins. Our observation that the ErbB3 protein was an excellent *in vitro* substrate for the EGF receptor (Figure 6), is consistent with the assumption that the documented *in vivo* phosphorylation of ErbB3 in response to either EGF or neuregulin results directly from the action of other ErbB family protein tyrosine kinases.

In summary, the catalytic capabilities of the ErbB3 receptor protein have been examined in this work. Neither a recombinant form of the predicted protein tyrosine kinase domain of ErbB3 nor the full-length protein immunoprecipitated from cells over-expressing the receptor displayed protein tyrosine kinase activity. The fluorescent nucleotide analog TNP-ATP, which binds with high affinity to the catalytic site of the EGF receptor protein tyrosine kinase, failed to interact with the recombinant ErbB3 protein. Hence the lack of intrinsic catalytic activity in ErbB3

might be attributed to a failure to bind ATP. ErbB3 was subsequently found to be an excellent substrate for the EGF receptor *in vitro*, which suggested that *in vivo* the ErbB3 protein can be transphosphorylated by other ErbB family protein tyrosine kinases.

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FOOTNOTES

Abbreviations used: EGF, epidermal growth factor; TNP-ATP, 2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; TKD, protein tyrosine kinase domain; hisTKD61, C-terminally complete EGF receptor cytosolic domain protein; hisTKD38, C-terminally truncated EGF receptor cytosolic domain; hisTKD-B3, ErbB3 cytosolic domain protein; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; Hepes, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid).

FIGURE LEGENDS

Figure 1 Schematic structures of recombinant EGF and ErbB3 receptor protein tyrosine kinase domains

The putative functional domains of the two receptor proteins are indicated: ligand binding domain (BD), transmembrane domain (TM), protein tyrosine kinase domain (TK), and autophosphorylation domain (AP). Candidate phosphorylation sites in the EGF receptor are identified, and YXXM labels the C-terminal domain of ErbB3 that contains seven repetitions of the consensus PI 3-kinase binding site, Tyr-Xaa-Xaa-Met. The Met-His-His-His-His-His leader peptide introduced into each of the recombinant proteins is also indicated (his₆).

Figure 2 Characterization of EGF receptor and ErbB3 TKDs by SDS-PAGE and circular dichroism spectroscopy

(A) Recombinant TKD proteins generated with the baculovirus/insect cell system were purified to near homogeneity by Ni²⁺-chelating column chromatography (see *Experimental Procedures*). SDS-PAGE analysis with silver staining of 0.5 µg samples of the three TKD forms is shown. (B) Circular dichroism spectra of EGF receptor (—) and ErbB3 (- - -) TKDs were recorded and analyzed for context of secondary structural elements (see *Experimental Procedures*). Percentages of α -helix, β -sheet, β -turn and random elements were 41%, 35%, 10%, and 14%, respectively, for the hisTKD61 protein, and 40%, 38%, 9%, and 13%, respectively, for the hisTKD-B3 protein.

Figure 3 Autophosphorylation and substrate phosphorylation activities of the EGF receptor and ErbB3 TKDs

Each of the TKD proteins (0.25 μ M) was incubated for 5 min at room temperature in the presence of 15 μ M [γ^{32} P]ATP, and either 10 mM MgCl₂ or 10 mM MnCl₂ as indicated. TKD autophosphorylation was analyzed by SDS-PAGE and autoradiography. Substrate phosphorylation activities of the TKD proteins were similarly assayed with the inclusion of 5 μ g of the protein substrate GST-TK7 in the incubation as indicated. The GST-TK7 protein shows multiple bands when phosphorylated.

Figure 4 Autophosphorylation activities of full-length EGF receptor and ErbB3 proteins

(A) Immunoprecipitation of ErbB3 and EGF receptor from cultured human breast cancer cell lines and NIH-3T3 cells expressing recombinant rat ErbB3 protein. Detergent lysates were prepared from NIH-3T3 fibroblasts transfected with the parent pcDNA3 expression vector (3T3-PC3) or the pcDNA3-ErbB3 vector (3T3-B3) and three human breast cancer cell lines: MDA-MB-453 (453), MDA-MB-468 (468), and SK-BR-3. Aliquots of each lysate (4 mg protein) were precleared with protein G-agarose, then immunoprecipitated with either ErbB3-specific antibody 2F12 (α -ErbB3) or EGF receptor-specific antibody LA1 (α -ER) as indicated. After washing twice, precipitates were resuspended and one half of each sample was analyzed by SDS-PAGE and immunoblotting with the immunoprecipitating antibodies. (B) Immune complex kinase assays performed with ErbB3 and EGF receptor immunoprecipitates. One fifth of each suspended immunoprecipitate analyzed in (A) was incubated for 10 min at room temperature in the presence of 17 μ M [γ^{32} P]ATP and either 10 mM MnCl₂ (lanes a) or a mixture of 10 mM MgCl₂

and 3 mM MnCl₂ (lanes b). The phosphoproteins were resolved by SDS-PAGE and identified by autoradiography.

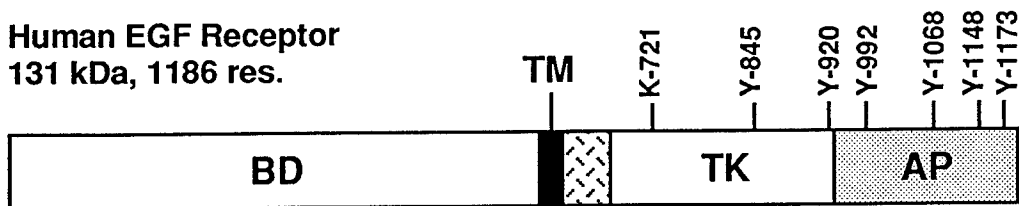
Figure 5 Nucleotide binding properties of recombinant EGF receptor and ErbB3 TKDs

The interaction of the nucleotide analog TNP-ATP with the recombinant TKD proteins was analyzed by fluorescence spectroscopy as previously described [21]. Whereas the EGF receptor-derived hisTKD61 protein (○) showed a high affinity interaction with TNP-ATP ($K_d = 0.75 \pm 0.24 \mu\text{M}$), the ErbB3-derived hisTKD-B3 protein (□) showed no interaction.

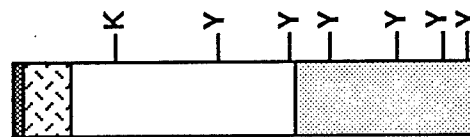
Figure 6 Autophosphorylation and cross-phosphorylation activities of EGF receptor and ErbB3 TKDs

(A) The C-terminally complete EGF receptor TKD (hisTKD61), the truncated EGF receptor TKD (hisTKD38), and the ErbB3 TKD (hisTKD-B3) (see Figure 1) (each at 0.25 μM concentration) were incubated either separately or together as indicated for 15 min at room temperature in the presence of 10 mM MnCl₂ and either 15 μM [$\gamma^{32}\text{P}$]ATP (left panel) or 15 μM ATP (right panel). Phosphorylation was analyzed by SDS-PAGE and autoradiography (left panel) or by antiphosphotyrosine immunoblotting (right panel). (B) The kinetics of phosphorylation of hisTKD-B3 by hisTKD38 were analyzed by incubating hisTKD38 (0.25 μM) in the presence of 0-2.5 μM hisTKD-B3 for 5 min at room temperature as in (A). ErbB3 phosphorylation was assayed by SDS-PAGE, autoradiography and scintillation counting of excised gel bands. The hyperbolic curve best fitting the experimental data is shown ($K_M=0.54 \mu\text{M}$; $V_{\text{max}}=1.4 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$).

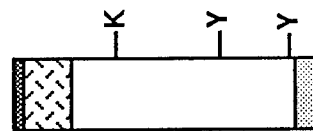
Human EGF Receptor
131 kDa, 1186 res.



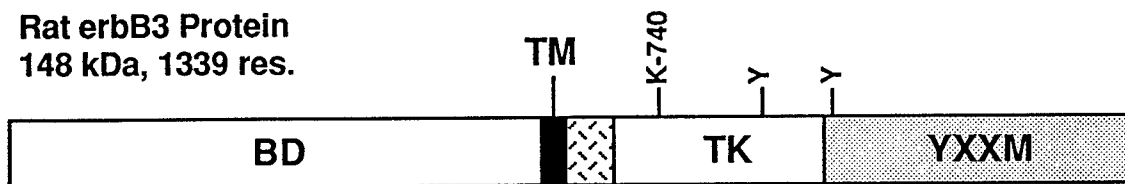
hisTKD61
61 kDa, 549 res.



hisTKD38
38 kDa, 335 res.



Rat erbB3 Protein
148 kDa, 1339 res.



hisTKD-B3
75 kDa, 681 res.

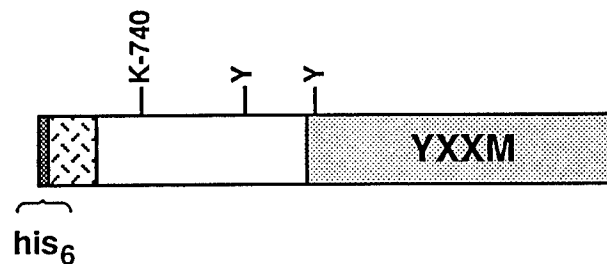


Figure 1

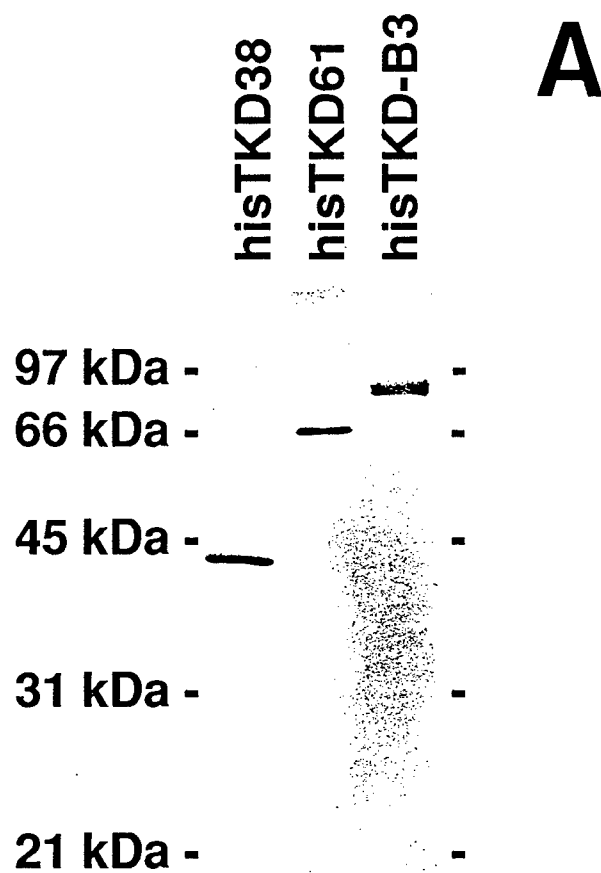


Figure 2A

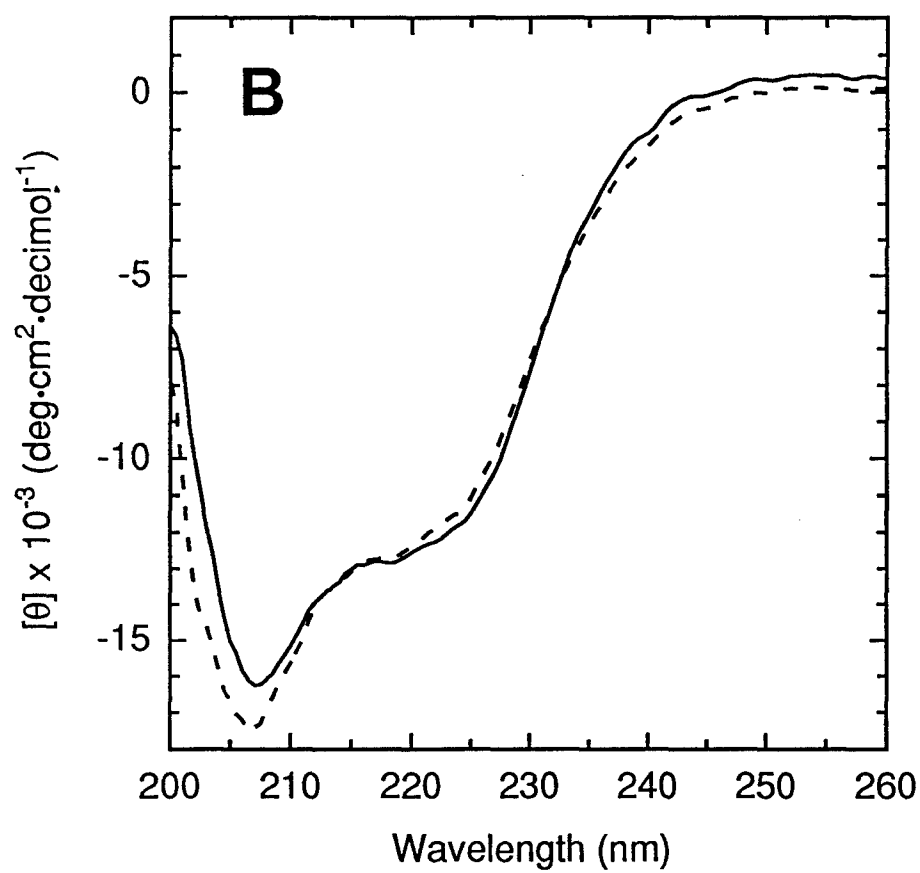


Figure 2B

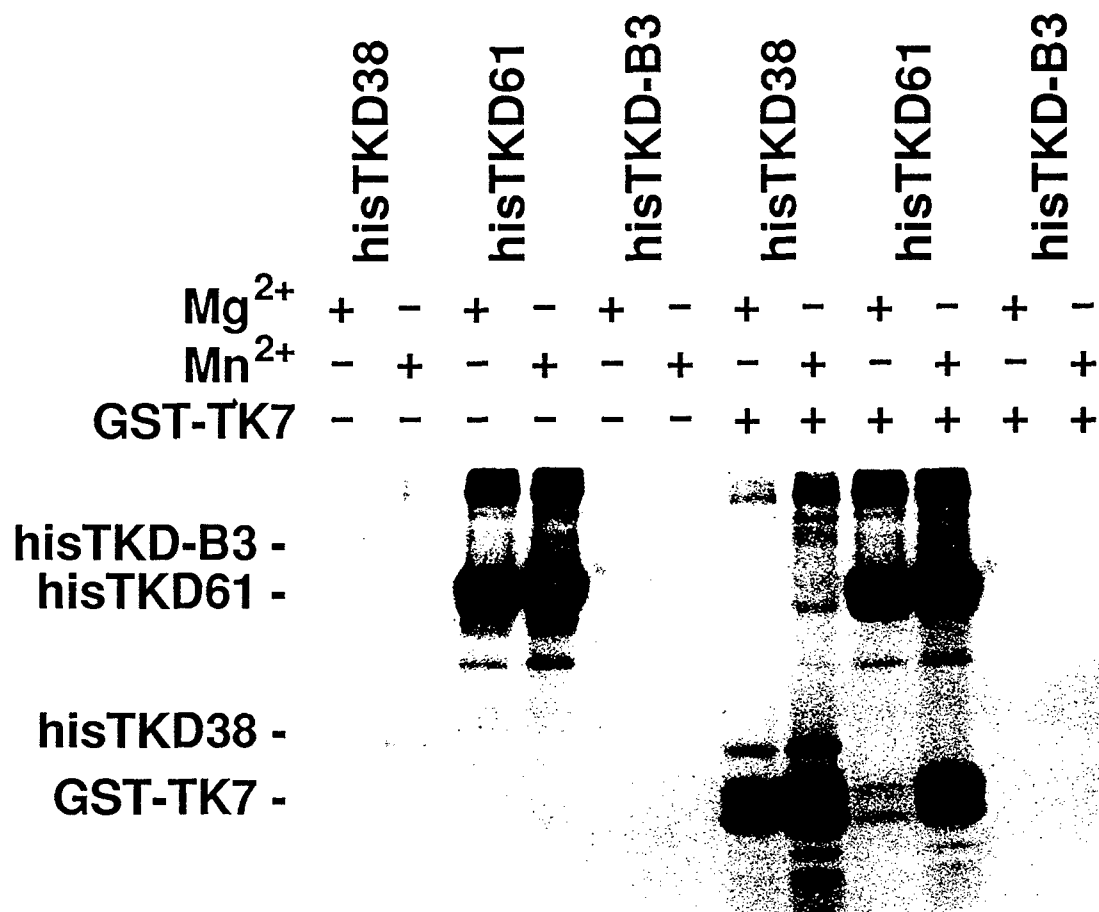


Figure 3



Figure 4A

B

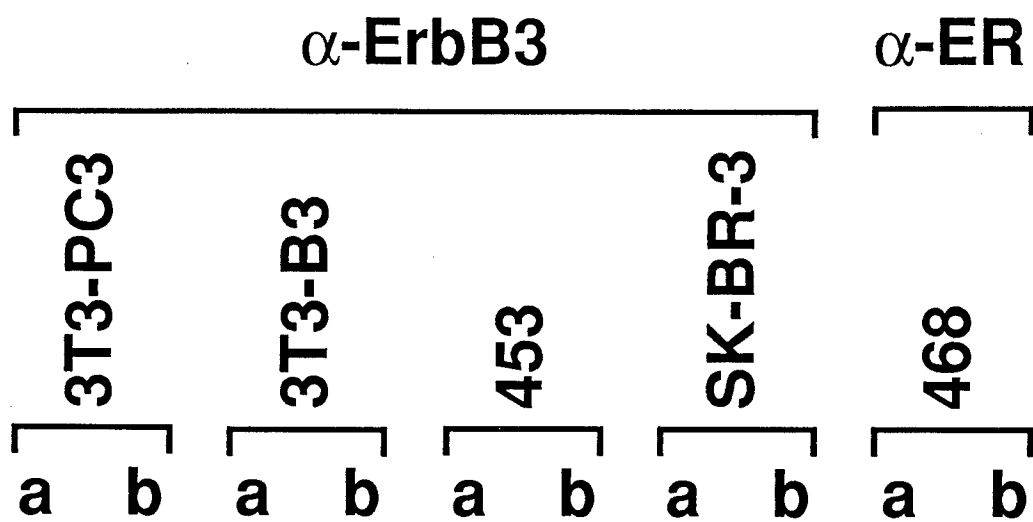


Figure 4B

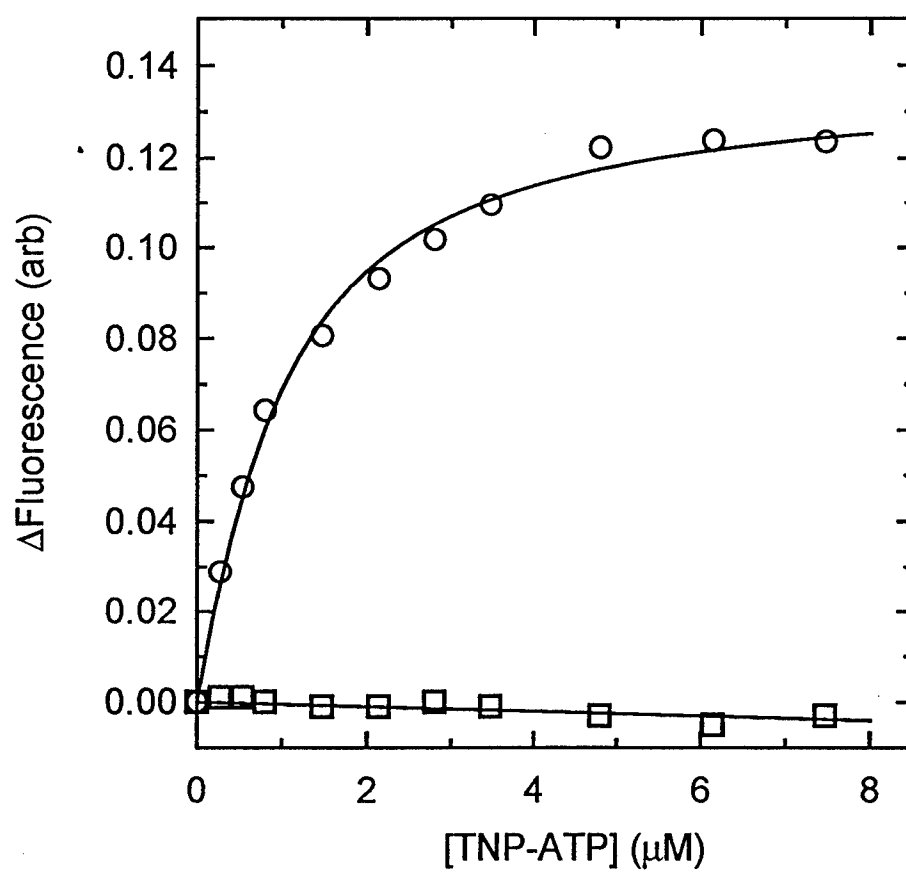


Figure 5

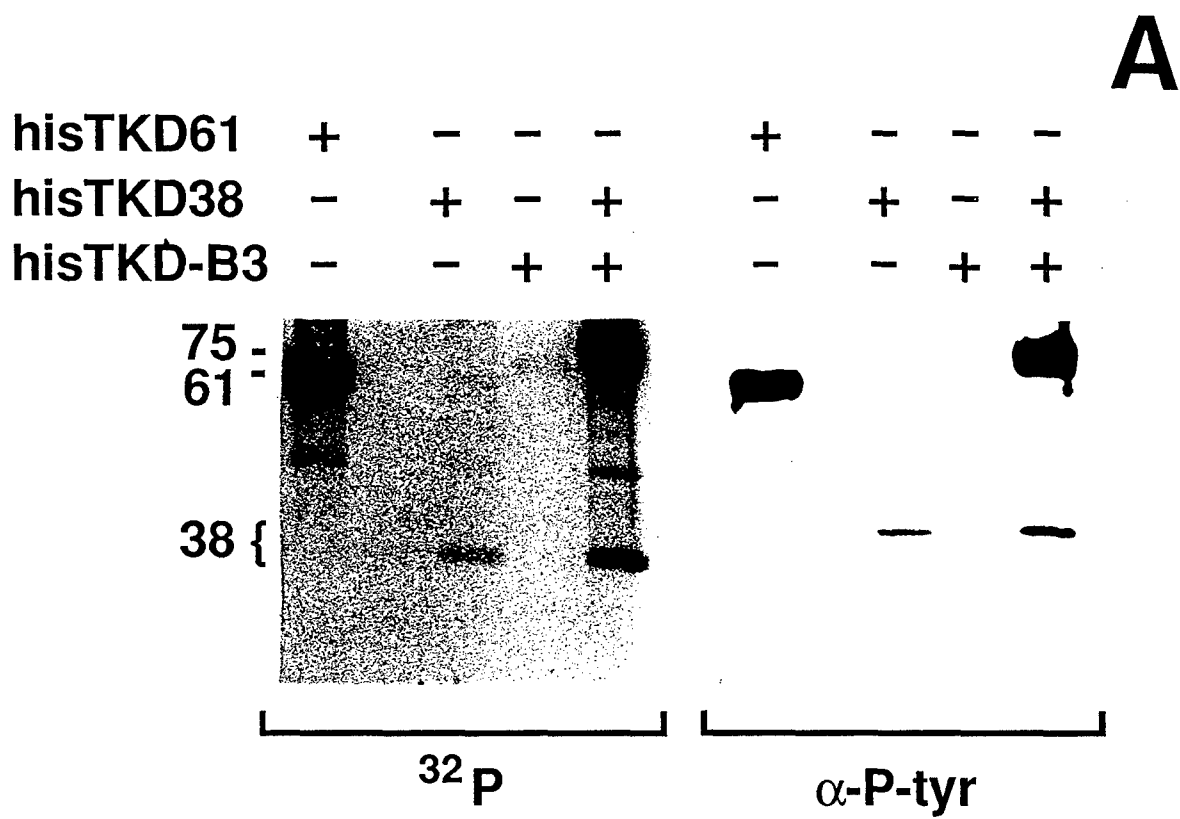


Figure 6A

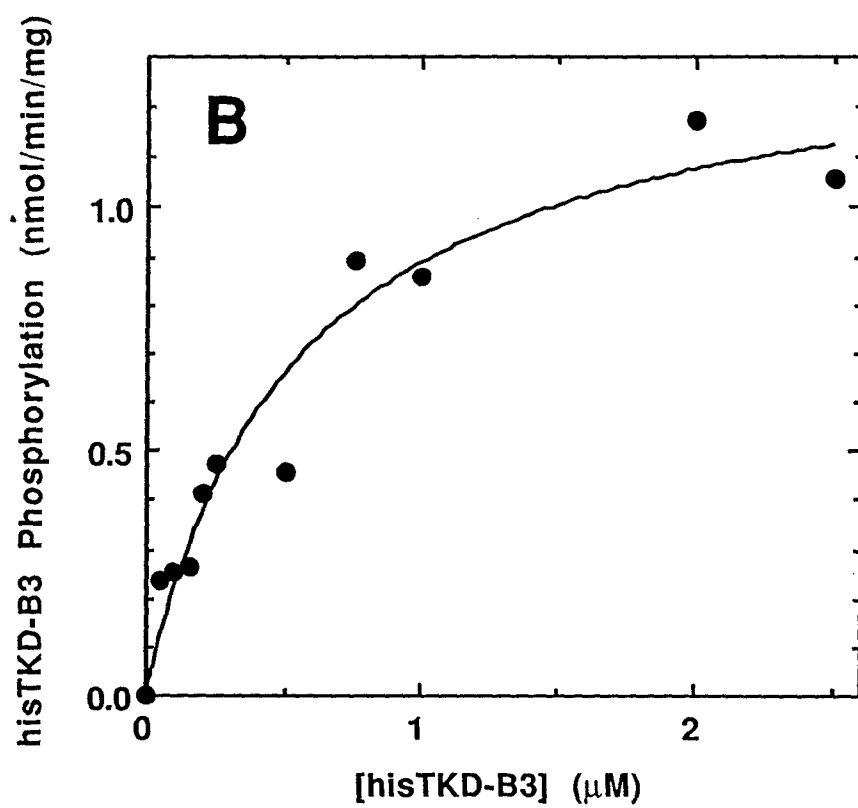


Figure 6B



DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information
Center, ATTN: DTIC-OCF, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

<u>Contract Number</u>	<u>Accession Document Number</u>
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DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864 ✓
DAMD17-94-J-4267	ADB216187 ✓
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DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management